Polymerase Chain Reaction-Restriction Fragment-Length Polymorphism Method to Distinguish *Liriomyza huidobrensis* from *L. langei* (Diptera: Agromyzidae) Applied to Three Recent Leafminer Invasions

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ABSTRACT A molecular method is presented for differentiating the morphologically cryptic leafminers *Liriomyza langei* Frick and *L. huidobrensis* (Blanchard). This method requires polymerase chain reaction (PCR) amplification of a 1031-bp region of mitochondrial cytochrome oxidase DNA followed by restriction fragment analysis using the restriction enzymes *Spe*I and *Eco*RV. *Spe*I cuts the mitochondrial fragment of *L. langei* into two fragments, but does not cut the *L. huidobrensis* fragment. *Eco*RV cuts the *L. huidobrensis* fragment into two fragments, but does not cut the *L. langei* fragment. This PCR-restriction fragment-length polymorphism (RFLP) method is faster and less costly than DNA sequencing, which is currently the only other way to differentiate these two species. We apply the method to samples from recently introduced leafminer populations in Sri Lanka, Canada, and South Africa and find that the invasive leafminer in all three locations is *L. huidobrensis.*

KEY WORDS Agromyzidae, polymerase chain reaction-restriction fragment-length polymorphism, molecular identification, pea leafminer, introduced species, invasive species

THE LEAFMINING FLIES *Liriomyza huidobrensis* (Blanchard) and *L. langei* Frick are important pests of a wide variety of vegetable and flower crops (Spencer 1973, Steck 1996). In 1973, Spencer (1973) synonymized *L. langei* with *L. huidobrensis* because of an apparent lack of morphological differences separating the two species. However, recent phylogenetic analyses of both mitochondrial and nuclear DNA sequence data have shown that these two groups of leafminers are substantially divergent in molecular characters and therefore represent morphologically cryptic species (Scheffer 2000, Scheffer and Lewis 2001). Scheffer and Lewis (2001) resuscitated the name *L. langei* for the cryptic species found in California and Hawaii and restricted the name *L. huidobrensis* to the cryptic species found in South and Central America.

Although *L. huidobrensis* and *L. langei* are endemic to the New World, since 1989 one or both of these species has spread to a number of additional regions including Europe (Cheek et al. 1993, Weintraub and Horowitz 1995), the Middle East (Weintraub and Horowitz 1995), and Asia (Shepard et al. 1996). Available evidence suggests that in most cases the invading flies are *L. huidobrensis* spreading from South and/or Central America. In some cases, importation of infested plant material from South or Central America has been implicated in new infestations (de Goffau 1991, Bartlett 1993). Additionally, analysis of both mitochondrial and nuclear DNA sequence data has unambiguously shown that samples from invasive populations in Israel, Indonesia, and Sri Lanka belong to *L. huidobrensis* rather than *L. langei* (Scheffer 2000, Scheffer and Lewis 2001). To date, *L. langei* has not been detected in any geographic location other than North America and Hawaii. However, given that it is only recently that this species could be distinguished from *L. huidobrensis,* it is possible that invasive populations of *L. langei* are present in other areas. Because *L. huidobrensis* and *L. langei* are suspected to differ in preferred hosts and in insecticide resistance status (Bartlett 1993, Weintraub and Horowitz 1995), it is important for management efforts that the species identity of newly introduced populations be known.

Currently, no morphological differences are known to differentiate *L. langei* from *L. huidobrensis,* although morphological studies are currently underway. Species identity can be readily determined using DNA sequence data from any of several mitochondrial and nuclear genes (Scheffer 2000, Scheffer and Lewis 2001), but this method is somewhat time-consuming and expensive for those not routinely involved with DNA sequencing.

The purpose of this article is to present a less expensive molecular method that can be used to rapidly differentiate *L. langei* from *L. huidobrensis.* This method uses the PCR combined with RFLP analysis

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Table 1. Source locality and host information for specimens for which mitochondrial sequence data (from Scheffer 2000) has indicated species identity (*L. langei* or *L. huidobrensis*)

N	Species	Country	Location	Host plant	Collector(s)
1	*L. langei*	United States	Monterey Co., CA	*Lactuca* sp. ("romaine")	Franklin Dlott, William Chaney
2	*L. langei*	United States	Monterey Co., CA	Lab Colony A	Franklin Dlott, William Chaney
2	*L. langei*	United States	Monterey Co., CA	Lab Colony B	Franklin Dlott, William Chaney
1	*L. langei*	United States	Monterey Co., CA	Lab Colony C	David Morgan, John Trumble
2	*L. langei*	United States	San Diego Co., CA	Lab Colony D	David Morgan, John Trumble
3	*L. langei*	United States	Hawaii, HI	*Apium* sp.	Robert Hollingsworth, Marshall Johnson
2	*L. langei*	United States	Maui, HI	*Allium* sp.	Laura Minuto, Ronald Mau
2	*L. huidobrensis*	Guatemala	Chimaltenango	*Pisum sativum*	Phillip Lamport, Steven Weller
2	*L. huidobrensis*	Guatemala	Chimaltenango	*Vicia fava*	Phillip Lamport, Steven Weller
1	*L. huidobrensis*	Guatemala	Chimaltenango	"weed in oak forest"	Phillip Lamport, Steven Weller
2	*L. huidobrensis*	Ecuador	Carchi	*Solanum* sp.	Roger Williams
1	*L. huidobrensis*	Sri Lanka	Galpalama	*Allium* sp.	Gamini Herath, Anura Wijesekara
1	*L. huidobrensis*	Sri Lanka	Galpalama	*Brassica oleracea*	Gamini Herath, Anura Wijesekara
1	*L. huidobrensis*	Sri Lanka	Sita Eliya	*Chrysanthemum* sp.	Gamini Herath, Anura Wijesekara
1	*L. huidobrensis*	Sri Lanka	Sita Eliya	*Brassica juncea*	Gamini Herath, Anura Wijesekara
2	*L. huidobrensis*	Israel	Gilat Exp. Station	*Solanum* sp.[a]	Phyllis Weintaub
1	*L. huidobrensis*	Israel	Gilat Exp. Station	*Apium* sp.[a]	Phyllis Weintaub
1	*L. huidobrensis*	Israel	Gilat Exp. Station	*Lactuca* sp.[a]	Phyllis Weintaub
1	*L. huidobrensis*	Indonesia, W. Java	Garut	*Solanum* sp.	Merle Shepard
2	*L. huidobrensis*	Indonesia, W. Java	Pangalengan	*Solanum* sp.	Merle Shepard

In the case of lab colonies, the location given is the geographic area from which the original colony founders were taken.
[a] Flies swept or vacuumed from host plant; all others reared.

(PCR-RFLP) of the mitochondrial cytochrome oxidase I and II region. We confirm the utility of this method by applying it to the specimens studied by Scheffer (2000) for which sequence data indicating species identity are available. We then use this method to determine the identity of additional specimens sampled from three recently introduced leafminer populations in Sri Lanka, South Africa, and Canada.

Materials and Methods

Acquisition of Specimens. Fly specimens were obtained from numerous hosts and locations around the world (Tables 1 and 2). Specimens used in this study included 31 individuals investigated by Scheffer (2000) (Table 1) as well as 52 additional specimens from invasive populations in Sri Lanka, South Africa, and Canada (Table 2).

Sri Lankan *L. huidobrensis*-type leafminers were first discovered in late 1996 in Nuwara Eliya, a semitropical region in the central hills located at an altitude of 6,000 feet In this region, a wide variety of vegetables are grown and leafminers were found on many crops at outbreak levels. Flies used in the study were reared from a number of hosts including cabbage, leeks, chrysanthemum, beets, mustard, and zucchini (Table 2).

South African *L. huidobrensis*-type leafminers were first discovered in late 1999 in the Sandveld region of the Western Cape. This region is primarily a potato growing area. In 2000 leafminers reached outbreak levels and the potato crop was greatly reduced. Flies

Table 2. Source locality and host information for specimens from invasive populations with unknown species identity[a]

N	Country	Location	Host Plant	Date	Collector(s)
3	Sri Lanka	Galpalama	*Beta vulgaris*	10.IX.1998	Gamini Herath, Anura Wijesekara
2	Sri Lanka	Galpalama	*Allium* sp.	10.IX.1998	Gamini Herath, Anura Wijesekara
2	Sri Lanka	Galpalama	*Brassica oleracea*	10.IX.1998	Gamini Herath, Anura Wijesekara
1	Sri Lanka	Sita Eliya	*Chrysanthemum* sp.	10.IX.1998	Gamini Herath, Anura Wijesekara
2	Sri Lanka	Sita Eliya	*Emilia son*	10.IX.1998	Gamini Herath, Anura Wijesekara
2	Sri Lanka	Sita Eliya	*Brassica juncea*	10.IX.1998	Gamini Herath, Anura Wijesekara
1	Sri Lanka	Magastota	*zuchini*	23.III.1998	Gamini Herath, Anura Wijesekara
13	South Africa	Sandveld, Western Cape	*Solanum tuberosum*	19.XI.1999	Diedrich Visser
3	South Africa	Sandveld, Western Cape	*Solanum tuberosum*	1.VIII.2000	Diedrich Visser
5	Canada	Simcoe Co., Ontario	(from two greenhouses)	22.II.2000	Andrea Martin
3	Canada	York Region, Ontario	(one greenhouse)	22.II.2000	Andrea Martin
4	Canada	Muck Res. Stat., Ont.	*Lactuca* sp., *Apium* sp.[a]	VIII.2000	Andrea Martin
2	Canada	Green Acre, Ontario	*Lactuca* sp., *Apium* sp.[a]	VII.2000	Andrea Martin
3	Canada	Simcoe Co., Ontario	greenhouse annuals[a]	VIII.2000	Andrea Martin
4	Canada	York Co., Ontario	greenhouse rose[a]	VIII.2000	Andrea Martin
2	Canada	Hillside	greenhouse celery[a]	VI.2000	Andrea Martin

[a] Flies swept or vacuumed from host plant; all others reared.

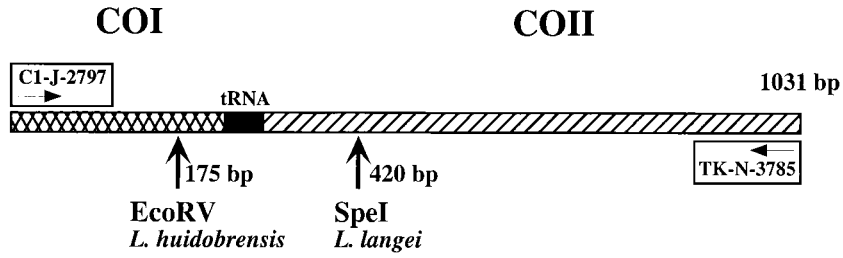

Fig. 1. Schematic diagram of the mitochondrial cytochrome oxidase fragment used in this study. Large arrows indicate the locations of the *Eco*RV recognition site (in *L. huidobrensis*) and the *Spe*I recognition site (in *L. langei*).

sampled in this study were reared in 1999 and 2000 from potato (Table 2).

Canadian *L. huidobrensis*-type leafminers specimens were first discovered in large numbers in southern Ontario in 1999 in both greenhouses and in field crops, although they were present in at least one greenhouse the previous year (Graeme Murphy, personal communication). In this region, a wide variety of vegetables and ornamentals are grown. Leafminers reached outbreak levels and some field crops suffered considerable damage. Flies used in this study were swept or aspirated from several crops in greenhouses and in the field (Table 2).

Molecular Methods. Methods of DNA extraction and PCR amplification were similar to those reported in Scheffer (2000). Fresh adults were preserved in 95–100% ethanol and stored at −80 C until DNA extraction. From single specimens, DNA was extracted using the insect protocol recommended by Qiagen (Valencia, CA). Primers C1-J-2797 (5′-CCTCGACGTTATTCAGATTACC-3′) and TK-N-3785 (5′-GTTTAAGAGACCAGTACTTG-3′) (Simon et al. 1994) were used to amplify a 1031 bp region of mitochondrial DNA spanning the 3′ region of COI, all of the leucine tRNA, and all of the COII gene. The conditions for PCR amplification using a Stratagene Robocycler (Stratagene, La Jolla, CA) were as follows: an initial denaturing step of 92°C for 2 min; 35 cycles of 92°C for 1 min 30 s, 50°C for 1 min 30 s, 72°C for 2 min 30 s; and a final extension step of 72°C for 7 min. Amplification products were purified using the QIAquick PCR Clean-Up Kit (Qiagen) and eluted in 30 μl of ultrapure water.

To identify restriction enzymes that could be used to distinguish *L. langei* from *L. huidobrensis*, sequences obtained previously (Scheffer 2000) were investigated for restriction recognition sites in the program Sequencher (Gene Codes, Ann Arbor, MI). *L. langei* was found to have a unique recognition site for *Spe*I, and *L. huidobrensis* was found to have a unique site for *Eco*RV (Fig. 1).

Restriction digests were performed using the restriction enzymes *Eco*RV and *Spe*I obtained from Gibco Life technologies (Gaithersburg, MD) in separate reactions following the manufacturer's protocol. Each purified amplification product was digested two separate times, once with each enzyme. The resulting DNA fragments were visualized on a 1.5% agarose gel along with a 100-bp DNA ladder (Gibco Life Technologies) to size fragments.

Restriction digests of mitochondrial amplification products from specimens sequenced previously by Scheffer (2000) were performed to confirm the utility of restriction analysis for determining species identity. Digests of new samples from Sri Lanka, Canada, and South Africa were performed to determine which of the two species is present in those areas.

Results

All 81 amplifications resulted in a single band of the appropriate length (1031 bp). *Spe*I restriction digests of the 13 known *L. langei* specimens ("California Clade," Scheffer 2000) all resulted in two discrete bands (representatives shown in Fig. 2a), indicating a single *Spe*I recognition site within the 1031 bp fragment. The precise lengths of these two bands are estimated from DNA sequence data from Scheffer (2000) to be one of length 420 bp and one of length 611 bp. *Spe*I restriction digests of the 18 known *L. huidobrensis* specimens ("South America Clade," Scheffer 2000) all resulted in a single band of the original length (Fig. 2a), indicating that in this species the 1031-bp fragment does not contain an *Spe*I recognition site.

Restriction digests with *Eco*RV exhibited the opposite pattern. *Eco*RV digests of fragments from the 18 *L. huidobrensis* specimens resulted in two bands (representatives shown in Fig. 2b), indicating a single *Eco*RV recognition site. The precise lengths of these two bands are estimated from DNA sequence data from Scheffer (2000) to be one of length 175 bp and the other of length 856 bp. The short band migrates quite quickly and is relatively faint on the gel (see arrow, Fig. 2b). Digests of the fragment from the 13 *L. langei* specimens resulted in the original band (Fig. 2b) and indicated no *Eco*RV recognition site within the fragment from this species. The 856 bp band from the *L. huidobrensis* digests migrates more rapidly than the 1031-bp band from *L. langei* (Fig. 2), and this difference can be made more apparent by using a lower concentration of agarose in the gel (e.g., 1.0% agarose).

The amplified fragment from all specimens from the invasive populations in Sri Lanka ($n = 17$), South Africa ($n = 16$), and Canada ($n = 23$) did not contain

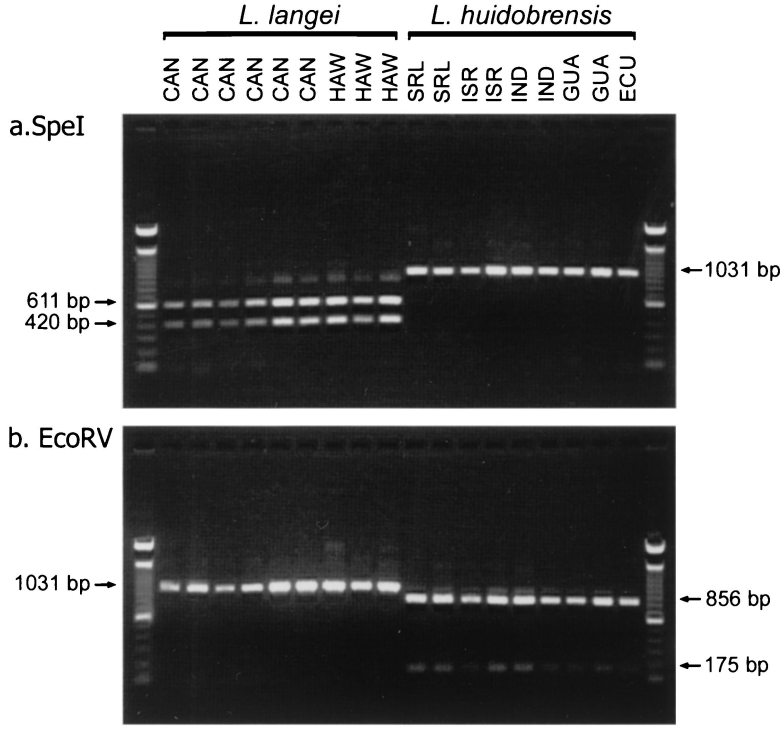

Fig. 2. Representative samples of *L. langei* and *L. huidobrensis* following restriction enzyme digests of mitochondrial cytochrome oxidase fragments using (a) *Spe*I or (b) *Eco*RV. CAN = Canada, HAW = Hawaii, SRI = Sri Lanka, IND = Indonesia, GUA = Guatemala, and ECU = Ecuador.

the *Spe*I recognition site (one band, Fig. 3a), but did contain the *Eco*RV sites (two bands, Fig. 3b), indicating that all sampled specimens belong to *L. huidobrensis.*

Discussion

Liriomyza huidobrensis and *L. langei* can be readily differentiated using the PCR-RFLP methodology described in this paper. Digestion of the mitochondrial fragment with either *Spe*I or *Eco*RV is enough to determine the species identity of a sample. However, the highest degree of accuracy with this method will be obtained when both restriction digests are performed. It is particularly important that identifications are not based solely on a negative result. For example, *Spe*I does not cut the *L. huidobrensis* fragment, and *Eco*RV does not cut the *L. langei* fragment. Because many factors can cause a restriction digest to fail (e.g., bad enzyme, poor reaction conditions) these negative results should not be used to indicate species affiliation. Identifications should only be made when a positive result has been obtained, i.e., when *Eco*RV has cut the *L. huidobrensis* fragment or when *Spe*I has cut the *L. langei* fragment.

The use of PCR-RFLP analysis for identification has several advantages over the use of DNA sequence data. Most importantly for general applicability, PCR-RFLP analysis can be performed by anyone with access to a lab containing a PCR thermocycler (and associated paraphernalia), an increasingly common piece of equipment. Other advantages are that PCR-RFLP analysis can usually be performed more quickly and at only a fraction of the cost of DNA sequencing. The main disadvantage of this method is that there is more opportunity for misidentification than with the use of sequence data. The loss of a restriction site only requires a single nucleotide change, and gaining a site may also only require a single nucleotide change, depending on the sequence of the adjacent nucleotides. It is possible that unsampled populations have, by chance, evolved these particular changes. However, we have sampled *L. langei* and *L. huidobrensis* from throughout their geographic ranges (additional *L. huidobrensis* sampled from various South American locations by S.J.S., unpublished data) and all can be correctly identified with the method described here.

Another complication with using PCR-RFLP analysis for identification is that it is appropriate to use the method only with those species for which the method was developed. Using our PCR-RFLP method with *L. bryoniae* (Kaltenbach), a close relative of *L. huidobrensis* and *L. langei*, will result in the incorrect identification of the specimen as *L. huidobrensis.* Fortunately, adult *L. bryoniae* can be identified using morphological characters (Spencer 1973).

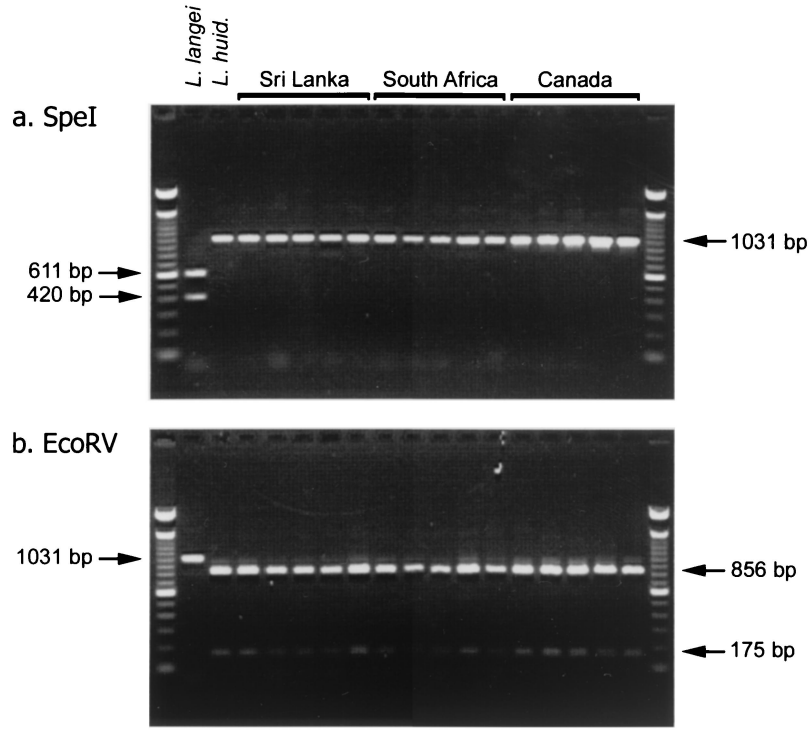

Fig. 3. Representative samples of invasive leafminer populations from Sri Lanka, South Africa, and Canada following restriction enzyme digests of mitochondrial cytochrome oxidase fragments using (a) *Spe*I or (b) *Eco*RV. One *L. langei* and one *L. huidobrensis* known from sequence data were run for comparison in lanes 2 and 3.

The PCR-RFLP identification method presented here can be used with adult, larval, or pupal specimens. However, the immature stages of many leafminer species are difficult to identify and inclusion of specimens not belonging to *L. langei* or *L. huidobrensis* could result in misidentifications as discussed above. Additionally, immature leafminers quite often contain hymenopteran parasitoids. The primers used here for DNA amplification are universal enough to have the potential to amplify hymenopteran DNA as well as fly DNA (Simon et al. 1994). The presence of hymenopteran DNA during restriction digests could give rise to anomalous and/or misleading restriction digest patterns. To avoid potential problems, working with adult fly specimens is best, but when this is not possible, larval or pupal specimens can be used with appropriate caution.

The PCR-RFLP method presented here was developed to provide researchers, pest managers and quarantine officials with a rapid and simple molecular method for accurately differentiating *L. langei* from *L. huidobrensis.* The PCR amplification is robust, even for those specimens that have not been optimally preserved. The restriction enzyme digests and subsequent interpretation are straightforward. The entire set of procedures, from DNA extraction to final identification, can be completed within a single working day.

To date, all invasive populations of this complex that have been investigated have belonged to *L. huidobrensis* rather than *L. langei* (Scheffer 2000, Scheffer and Lewis 2001). The current study increases the sampling and confirms previous results regarding the identity of the invasive population in Sri Lanka (Scheffer 2000). We also document for the first time the presence of this species in South Africa and in Canada. The global spread of *L. huidobrensis* appears to be coincident with the evolution of resistance to numerous insecticides by this species and a corresponding change in its status from being a relatively unimportant secondary pest to a highly damaging threat (Weintraub and Horowitz 1995). Given the increasing importance of global trade, it is expected that this species will continue to spread to new locations. However, it should not yet be concluded that all invasive *L. huidobrensis*-type leafminers are, in fact, *L. huidobrensis. L. langei* is currently an increasingly destructive pest in California (Chaney 1995, Morgan et al. 2000), and additional sampling may find that *L. langei* has also spread to new locations. The current study provides a quick and relatively inexpensive molecular method for researchers and pest managers to confirm the identity of the populations they study.

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